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1.0 Purpose

The purpose of this assay is to determine the bioactivity of rhIL-7 by using 2E8 cell proliferation assay.

2.0 Scope

This SOP is to be used for qualitative and quantitative measurement of the 2E8 cell proliferation by rhIL-7 and to compare bioactivity of different lots of products relative to a reference lot and also to monitor stability of rhIL-7 product.

3.0 Authority and Responsibility

3.1 The Director, Process Analytics/Quality Control (PA/QC), and the Manager, Biopharmaceutical Development Program (BDP) have the authority to define this procedure.

3.2 The Manager, BDP, is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA).

3.3 BDP PA/QC personnel are responsible for the performance of this procedure.

3.4 BDP PA/QC personnel are responsible for reviewing the data and documentation of the results of this procedure.

3.5 BQA is responsible for quality oversight of this procedure.

4.0 Equipment and Accessories Required

4.1 CO_2 Incubator, Forma Scientific, Inc., or equivalent

4.2 Biological safety cabinet, Baker (Model B6-0001), or equivalent

4.3 Water bath

4.4 Hemocytometer

4.5 Microscope

4.6 ELISA Plate Reader (SPECTRA MAX190 from Molecular Devices or equivalent) and Software (Softmax Pro or equivalent)

4.7 96 well cell culture cluster, BDP PN 20050, or BDP approved equivalent

4.8 Pipetman 0.5-10 uL, 2-20 uL, 10-100 uL, 20-200 uL and 100-1000 uL, (Rainin or VWR, or equivalent)

4.9 Multi-channel pipettes (8 and 12 channels)

4.10 Disposable pipettes 5 mL (BDP PN 20104), 10 mL (BDP PN 20100), 25 mL (BDP PN 20102), 50 mL (PN 20105) or BDP approved equivalent

4.11 Pipet Tips 10 μL (BDP PN 20738), 250 μL (BDP PN 21767), 1000 μL (BDP PN 20769), or BDP approved equivalent

4.12 Reagent Reservoir, 50 mL, BDP PN 20481, or BDP approved equivalent

4.13 Eppendorf tube, 1.5 mL (BDP PN 20659), Cryovial tubes (BDP PN 20007), or BDP approved equivalent

4.14 Filter (0.2 μm), BDP PN 20184, or BDP approved equivalent

4.15 Cell culture flask, T-25 (BDP PN 21611), T-75 (BDP PN 21610), T150 (BDP PN 21603) or BDP approved equivalent

4.16 Disposable centrifuge tubes, 15 mL (BDP PN 20006), 50 mL (BDP PN 20140), or BDP approved equivalent

4.17 Centrifuge, Beckman Coulter or equivalent

4.18 Refrigerator (2º-8°C), Thermo Electron or equivalent

4.19 Freezer (-20°C), Revco or equivalent

4.20 Freezer (-65°C or below), Thermo Electron or equivalent

4.21 Plate shaker

5.0 Reagents

5.1 IMDM medium (with L- Glutamine), BDP PN 30977, or BDP approved equivalent

5.2 Fetal Bovine Serum, Defined, BDP PN 30891, or BDP approved equivalent

5.3 Mouse Interleukin-7 (mIL-7) for tissue culture, BDP PN 30980 or BDP approved equivalent

5.4 MTS: Cell titer 96 Aqueous One Solution Cell Proliferation Assay, BDP PN 30546 or BDP approved equivalent

5.5 Milli-Q H_2O, In-house preparation

5.6 Trypan Blue, BDP PN 10095, or BDP approved equivalent

5.7 PBS, BDP PN 30007, or BDP approved equivalent

5.8 Hank's buffered salt solution (HBSS), BDP PN 30656, or BDP approved equivalent

5.9 rhIL-7 Reference Standard

5.10 SDS solution (10%), BDP PN 30532, or BDP approved equivalent

5.11 2-Mecrcaptonethanol, BDP PN 30588 or BDP approved equivalent

6.0 Cells and Cell Culture

NOTE: Perform all steps aseptically in a certified BSC; follow ***SOP 22909 - Use, Cleaning and Disinfection of Equipment and Laboratories in PA/QC***. For cell recovery, propagation, medium preparation and documentation, follow ***SOP 22140 - Mammalian Cell Culture - Initiation and Maintenance of Cell Cultures in Process Analytics/Quality Control***. The volume of medium, samples, or reagents should be within ± 5% of indicated volume as below unless specified.

6.1 Cell Line 2E8, ATCC TIB-239 (B lymphocyte; mouse). The 2E8 cell bank (BDP PN 10571) was generated by the BDP Cell Culture Lab from cells obtained from ATTC. Details of cell bank generation and testing are captured in MPR-C-17 for Lot [redacted] frozen on 4/26/2010 and QC-044892 (Sterility) and QC-044893 (mycoplasma) testing.

6.2 2E8 Cell **Culture Medium**

Reagent	Amount
IMDM medium (5.1)	400 mL
FBS (5.2)	100 mL
mIL-7 (5.3)	4 ng/mL
2-Mercaptoethanol (5.11)	0.05 mM

NOTE: mIL-7 is added (under sterile conditions) directly into the cell culture flask immediately before use or complete media just before use.

Label the complete medium as 2E8 Cell Culture Medium with the lot number, date prepared, initials, and expiry date based on the component with the shortest expiration date. Store at 2º - 8ºC.

6.3 Preparation of 2E8 Cell **Assay Medium**

Reagent	Amount
IMDM medium (5.1)	450 mL
FBS (5.2)	50 mL
2-Mercaptoethanol (5.11)	0.05 mM

Label as 2E8 Cell Assay Medium with the lot number, date prepared, initials, and expiry date based on the component with the shortest expiration date. Store at 2º - 8ºC.

6.4 Cell Line 2E8, ATCC TIB-239 (B lymphocyte; mouse). The 2E8 cell bank (BDP PN 10571) was generated by the BDP Cell Culture Lab from cells obtained from ATTC. Details of cell bank generation and testing are captured in MPR-C-17 for Lot [redacted] frozen on 4/26/2010 and QC-044892 (Sterility) and QC-044893 (mycoplasma) testing

6.5 2E8 Cell **Culture Medium**

Reagent	Amount
IMDM medium (5.1)	400 mL
FBS (5.2)	100 mL
mIL-7 (5.3)	4 ng/mL
2-Mercaptoethanol (5.11)	0.05 mM

NOTE: mIL-7 is added (under sterile conditions) directly into the cell culture flask immediately before use or complete media just before use.

Label the complete medium as 2E8 Cell Culture Medium with the lot number, date prepared, initials, and expiry date based on the component with the shortest expiration date. Store at 2º - 8ºC.

6.6 Preparation of 2E8 Cell **Assay Medium**

Reagent	Amount
IMDM medium (5.1)	450 mL
FBS (5.2)	50 mL
2-Mercaptoethanol (5.11)	0.05 mM

Label as 2E8 Cell Assay Medium with the lot number, date prepared, initials, and expiry date based on the component with the shortest expiration date. Store at 2º - 8ºC.

6.7 Cell Recovery and Passage

Follow ***SOP 13209 - Mammalian Cell Culture - Initiation and Maintenance of Cell Cultures***.

6.8 Cell Count and Cell Viability

Follow ***SOP 13214 - Using a Hemocytometer to Determine Density, Viability, Generation Time and Doubling Time for Mammalian Cells***.

7.0 Assay Procedure

NOTE: For CGMP product release assay must be performed in triplicate plates with the test article and reference standard in each plate. The CV% for the ED_{50} values and ED_{50} ratio for standard and test article should be within ± 30% of the mean. The average of the three ED_{50} values and average ratio are reported as the result. The assay for stability tests or non-GMP tests can be run in a single plate.

7.1 Perform cell count and cell viability following 6.5. Cell viability has to be ≥ 65%.

7.2 Wash the cells two times with HBSS (1000 rpm, 10 minutes) and adjust the cell concentration to a density of 2 x 10^6 cells/mL (viable cells) using assay medium. Incubate the cells at 37º ± 2°C in 5 ± 1% CO2 and ≥ 70% humidity air atmospheres for 1 ± 0.5 hours.

NOTE: Cells at the passages between 3 to 8 must be used in GMP assays.

7.3 During the cell incubation period, prepare rhIL-7 Reference and test sample working solutions.

7.3.1 **Dilute the Reference Standard to an initial dilution (DF1) of 10 μg/mL**

Calculate the volume of dilution buffer (**Vs**) as:

Vs = [Concentration of Reference Standard (μg/mL) × 10 μL ÷ 10 μg/mL]-10μL

7.3.2 Take 10 μL of Reference Standard and **Vs** μL of PBS. Mix well and label as **DF1**.

7.3.3 Dilute the DF1 to the second dilution of 200 ng/mL: Add 20 μL of the DF1 to 980 μL of assay medium, mix well and label as **Std01**.

7.4 Preparation of Test Sample working solution

Sample dilution is dependent on protein concentration of the sample. The sample dilution is to be prepared in the same way as the reference lot. The starting dilution is labeled as sample01. A titration curve covering the entire standard rhIL-7 is generated from target range of 200 ng/mL to 0.01 ng/mL.

7.5 The Reference Standard and test sample working solutions can be run in series dilution (1:3) in an empty plate. The following is an example for dilution details to use as a reference:

7.5.1 Using a 50-300 multiple-channel pipette, add the assay medium into the columns 1-10 of the plate at 100 μL/well.

7.5.2 Using a 50-300 multiple-channel pipette, add the Starting Std01 or sample 01 into the column 11 at 150 μL/well in triplicate.

7.5.3 Using a 5-50 multiple-channel pipette, take 50 μL/well from the column 11 into the column 10 in triplicate and mix well by pipetting up and down 5 times.

7.5.4 Using a 5-50 multiple-channel pipette, take 50 μL/well from the column 10 into the column 9 in triplicate and mix well by pipetting up and down 5 times.

7.5.5 Using a 5-50 multiple-channel pipette, take 50 μL/well from the column 9 into the column 8 in triplicate and mix well by pipetting up and down 5 times.

7.5.6 Using a 5-50 multiple-channel pipette, take 50 μL/well from the column 8 into the column 7 in triplicate and mix well by pipetting up and down 5 times.

7.5.7 Using a 5-50 multiple-channel pipette, take 50 μL/well from the column 7 into the column 6 in triplicate and mix well by pipetting up and down 5 times.

7.5.8 Using a 5-50 multiple-channel pipette, take 50 μL/well from the column 6 into the column 5 in triplicate and mix well by pipetting up and down 5 times.

7.5.9 Using a 5-50 multiple-channel pipette, take 50 µL/well from the column 5 into the column 4 in triplicate and mix well by pipetting up and down 5 times.

7.5.10 Using a 5-50 multiple-channel pipette, take 50 µL/well from the column 4 into the column 3 in triplicate and mix well by pipetting up and down 5 times.

7.5.11 Using a 5-50 multiple-channel pipette, take 50 µL/well from the column 3 into the column 2 in triplicate and mix well by pipetting up and down 5 times and pipette out 50 µL/well sample from column 2 to waste.

NOTE: Alternatively, for the non-GMP requests with more samples, the Reference Standard and samples can be run in series dilution from row to row covering the different concentration range in an empty plate. Document the actual dilution details and template in data printout.

7.6 After the 1 ± 0.5 h incubation of the cells in step 7.2, transfer cell suspension into the wells of the plate containing rhIL-7, 100 µL/well. Keep at least three wells containing cells without test articles as cell controls for each sample (rhIL-7 concentration = 0). The final concentration target range is from 100 ng/mL to 0.005 ng/mL.

7.7 Add 200 µL/well of the assay media into all empty wells as Blank controls.

7.8 Incubate above plate in an incubator at 37 ± 2°C in 5 ± 2% CO2 and ≥ 70% humidity air atmospheres for 48 ± 2 hours.

7.9 Using a multiple-channel pipette, add 20 µL/well of MTS solution (5.4) and incubate the plate for an additional 4 ± 0.5 hours at 37ºC in a CO_2 incubator.

7.10 Add 10% SDS solution (5.10) 25 µL/well and shake for 1 minute on a titer plate shaker at room temperature.

7.11 Read the plate at 490 nm using a microplate reader as per ***SOP 16144 - Operation of SpectraMax Series (190, 384 plus, M2, M5e, etc.) Plate Reader (Molecular Device)***.

7.12 Print out the raw data and template and attach them to the QC Test Request or date file. Initial, date, label with QC Test Request number.

8.0 Data Analysis and Acceptance Criteria

8.1 Data analysis is performed using the 4-parameter curve fit as described in the Instruction Manual of the instrument supplier. For estimation of test sample titer relative to that of the reference standard, the reference sample block is labeled standards and the protein values are entered in the template for standard series. Test sample blocks are labeled unknown dilution factors and entered in the template. Standard curve is plotted and the curve fit is selected both for Graph and the standard curve. A 4-parameter fit analysis is performed using standard curve constructed with concentration on X-axis and Mean ODs at Y-axis with error Stdev. Alternate modes of analysis may be performed and explained if required.

8.2 For the Reference Standard, the following criteria should be met for the assay acceptance or validity:

8.2.1 Blank Controls OD (media only) must be ≤ 0.6.

8.2.2 Cell Control readout (cells only without rhIL-7) must be ≤ 0.3 (Blank background corrected reading).

8.2.3 Maximal OD must be ≥ 0.70 (background corrected reading).

8.2.4 The CV% of OD readout for any set of dilution in the range used for calculation (the steep or smooth region of the 4-parameter curve) must be within 25%.

8.2.5 R^2 for curve fit must be ≥ 0.985.

If these criteria are not met or abnormal observation in 4-parameter dose-dependent curve is observed, discuss with the Supervisor.

8.3 Bioactivity of test articles will be calculated by ED_{50} ratio and back-calculation mode and expressed in ng/mg under the assay conditions as well as ng/mg based on standard curve generated using rhIL7 Reference Standard.

8.3.1 For ED_{50} ratio-calculation mode, the activity of test samples will be calculated using the formula: Relative activity = (ED_{50} of reference lot ÷ ED_{50} of test article) x 100%

8.3.2 For back-calculation mode, the activity of test samples will be calculated from the OD read-out of known dilutions by extrapolation to the 4-Parameter curve fit standard curve generated from Reference Lot on X-axis based on the assigned activity or protein concentration value. The test article will be treated as sample of unknown concentration and activity extrapolation will be based on the absorbance reading of test articles extrapolated to standard curve. Values adjusted for all sample dilutions with absorbance in the steep part of the standard curve absorbance reading will be averaged (Do not use sample dilutions whose OD readings are in the plateau or apex.) and reported as the activity of the test article. Document calculations on data printout. Relative activity of test article = (estimated activity ÷ reported protein concentration) x 100%.

9.0 Documentation

9.1 All protocols, raw data, computer records, completed Forms 16140-01 and 16140-02, and the original copy of the final report will be maintained by BQA Documentation. Generate and maintain all documentation relevant to this SOP according to ***SOP 21409 - Good Documentation Practices***.

10.0 References and Related Documents

SOP 13209 *Mammalian Cell Culture - Initiation and Maintenance of Cell Cultures*

SOP 13214 *Using a Hemocytometer to Determine Density, Viability, Generation Time and Doubling Time for Mammalian Cells*

SOP 21409 *Good Documentation Practices*

SOP 22140 *Mammalian Cell Culture - Initiation and Maintenance of Cell Cultures in Process Analytics/Quality Control*

SOP 22909 *Use, Cleaning and Disinfection of Equipment and Laboratories in PA/QC.*

SOP 16144 *Operation of SpectraMax Series (190, 384 plus, M2, M5e, etc.) Plate Reader (Molecular Device)*

Form 16140-01 Measurement of rhIL-7 Bioactivity Using 2E8 Cell Proliferation Assay

Form 16140-02 Reagents, Materials and Equipment

Operating Procedure for the operation of ELISA Plate Reader (Molecular Device Instruction Manual for Softmax190 and Softmax 384 plus).